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# Cloning, Physical Mapping and Expression of Chromosomal Genes Specifying Degradation of the Herbicide 2,4,5-T by 'Pseudomonas cepacia' AC1100

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# Cloning, physical mapping and expression of chromosomal genes specifying degradation of the herbicide 2,4,5-T by *Pseudomonas cepacia* AC1100

(Recombinant DNA; Tn*5* mutagenesis; 2,4,5-trichlorophenoxyacetic acid; 5-chloro-1,2,4-trihydroxybenzene; chlorohydroxy-hydroquinone; 5-chloro-2-hydroxy-1,4-benzoquinone; isolation of degradative gene; homology; phage λ)

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SUMMARY

A genomic library of total DNA of *Pseudomonas cepacia* AC1100 was constructed on a broad-host-range cosmid vector pCP13 in *Escherichia coli* AC80. A 25-kb segment was isolated from the library which complemented a Tn*5*-generated, 2,4,5-trichlorophenoxyacetic acid-negative (2,4,5-T$^-$) mutant, *P. cepacia* PT88. This mutation was partially characterized and appeared to be lacking functional enzyme required for metabolism of an intermediate of the 2,4,5-T pathway, recently identified as 5-chloro-1,2,4-trihydroxybenzene [Chapman et al., Abstr. Soc. Environ. Toxicol. Chem. USA 8 (1987) 127]. A simple colorimetric assay was developed to detect the presence of this active enzyme in intact cells and was used to determine the expression of complementing genes. Subcloning experiments showed that a 4-kb *Bam*HI-*Pst*I fragment and a 290-bp *Pst*I-*Eco*RI fragment, separated by 1.3-kb, were required for complementation. Both fragments are identified to be chromosomal in origin. Hybridization studies using the subcloned fragments revealed that in addition to a Tn*5* insertion, mutant PT88 contained an extensive chromosomal deletion accounting for its 2,4,5-T$^-$ phenotype. The cloned fragments did not show homology to plasmid DNAs carrying degradative genes for toluene, naphthalene and 3-chlorobenzoate.

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Abbreviations: Ap, ampicillin; BSM, basal salts medium; bp, base pair(s); CHQ, chlorohydroxyhydroquinone; 2,4-D, 2,4-dichlorophenoxyacetic acid; ET, see MATERIALS AND METHODS, section e; EtdBr, ethidium bromide; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria-Bertani broth; Km, kanamycin; kb, 1000 bp; R, resistance; SDS, sodium dodecyl sulfate; Tc, tetracycline; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; wt, wild type; [ ], designates plasmid-carrier state.

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## INTRODUCTION

*Pseudomonas cepacia* AC1100, developed under strong selective pressure in a chemostat, is capable of growth and complete mineralization of 2,4,5-T (Kellogg et al., 1981). The precise mode of recruitment of the genes encoding 2,4,5-T degradation could not be confirmed because of a limited knowledge of the genetics and biochemistry of 2,4,5-T degradation. Although Ghosal et al. (1985b) found that AC1100 harbors at least two plasmids and demonstrated a considerable homology of one of these plasmids with a fragment of 2,4-D-degradative plasmid, pJP4 (Don and Pemberton, 1983), exact involvement of plasmids in 2,4,5-T degradation is not fully understood. Tomasek et al. (1986) detected Tn*5* insertions in the chromosomal DNA of two different Tn*5*-generated mutants with a 2,4,5-T$^-$ phenotype. To understand the precise roles of plasmid and chromosomal genes in the degradation of 2,4,5-T by strain AC1100, we sought to construct a gene bank of AC1100 and characterize the genes which are directly involved in 2,4,5-T degradation. In this paper, we report cloning, physical mapping and expression of one of the chromosomal genes of AC1100 playing a key role in the utilization of 2,4,5-T.

## MATERIALS AND METHODS

### (a) Bacterial strains, plasmids and growth media

Genotypes and sources of bacterial strains and plasmids are listed in Table I. All mutants of AC1100 used in this study were isolated by Tn*5*-insertion mutagenesis as reported earlier (Tomasek et al., 1986). *E. coli* strains were grown on LB medium (Maniatis et al., 1982). For cultivation and growth studies of *P. cepacia*, a BSM containing 2,4,5-T (Aldrich, 1 mg/ml) or glucose (0.2%) as sole carbon source was used (Kilbane et al., 1982). For solid media, agar (Difco) was added at a concentration of 1.5%. *Pseudomonas* isolation agar (Difco) was used to maintain transconjugants of *P. cepacia* mutants. Antibiotics were used in selective media at the following concentrations: Tc, 10 μg/ml for *E. coli* and 100 μg/ml for *Pseudomonas*; Km, 25 μg/ml for *E. coli* and 50-100 μg/ml for *Pseudomonas*; Ap, 100 μg/ml for *E. coli*.

### (b) Assay of gene expression

Cells of *P. cepacia* AC1100 and its mutants were grown on glucose (uninduced) or on glucose plus 2,4,5-T (induced) for 15 h. Cells were washed and suspended in 0.05 M phosphate buffer (pH 7.2). For determining the accumulation of intermediates, washed cells of the 2,4,5-T$^-$ mutant, PT88, were adjusted to $A_{540} = 2$ in incubation mixtures containing 0.1 to 1 mM of substrate. The ability to convert 4,6-dichlororesorcinol to 5-chloro-1,2,4-trihydroxybenzene by PT88 cells and its subsequent autooxidation to a colored chlorohydroxy-1,4-benzoquinone ($\lambda_{max} = 493$ nm) was used as the basis of an assay to determine the expression of AC1100 genes complementing the PT88 mutation. Washed cells of PT88 alone or carrying cloned fragments were incubated with 1 mM 4,6-dichlororesorcinol (Aldrich Chem. Co., Milwaukee, WI) at 30°C and the time course of absorption at 493 nm was followed with reaction supernatants. Dry weight of washed cells was determined from a standard curve established for AC1100.

### (c) Isolation of AC1100 genomic DNA

Total DNA of AC1100 was obtained by a modification of a procedure of Meade et al. (1982). AC1100 cells from 250-ml cultures grown on BSM with 2,4,5-T (1 mg/ml) as the sole carbon source were pelleted by centrifugation, washed twice in saline and suspended in 10 ml of ET buffer (10 mM EDTA, 10 mM Tris · HCl, pH 8.0) and used for total DNA isolation.

### (d) Isolation of plasmid DNA

Plasmid DNA from *E. coli* was prepared by a Triton X-100 lysis procedure, followed by EtdBr-CsCl equilibrium density gradient centrifugation (Guerry et al., 1973). The method used for rapid small-scale isolation of plasmid DNA was a modification of the boiling method (Holmes and Quigley, 1981). Plasmid DNA from *P. cepacia* AC1100 and its mutants was isolated by a procedure based on methods developed by Casse et al. (1979). A wet

TABLE I

Bacterial strains and plasmid vectors

| Strains or plasmids | Genotype, phenotype or description [a] | Source or reference |
|---|---|---|
| **Strains** | | |
| *E. coli* K-12 | | |
| AC80 | *thr*, *leu*, *met*, *hsdR*, *hsdM* | Chakrabarty et al (1978) |
| HB101 | *recA*13, *ara*14, *galK*2, *lacY*1, *mtl*1, *xyl*5, *proA*2, *hsdS*20, *rpsL*20 ($Sm^R$), *glnV*44 | Boyer and Roulland-Dussoix (1969) |
| JM83 | *ara*, *Δ(lac-proAB)*, *rpsL*($Sm^R$) *thi*(*φ*80d*lacZ ΔM*15) | Deretic et al. (1987) |
| *P. cepacia* | | |
| AC1100 | 2,4,5-$T^+$ wt | Kilbane et al. (1982) |
| PT88 and other Tn*5*-induced mutants | AC1100::Tn*5*, 2,4,5-$T^-$, $Km^R$ | Tomasek et al. (1986) |
| **Plasmids** | | |
| pRK290 | IncP1, *mob*$^+$, *tra*$^-$, $Tc^R$ | Ditta et al. (1980) |
| pVDZ'2 | IncP1, *mob*$^+$, *tra*$^-$, *lacZ*, $Tc^R$ | Deretic et al. (1987) |
| pCP13 | $Tc^R$, $Km^R$, *ccs*$^+$ | Friedman et al. (1982); Darzins and Chakrabarty (1984) |
| pRK2013 | ColE1, *mob*$^+$, *tra*$^+$ (RK2), $Km^R$ | Figurski and Helinski (1979) |
| pMMB6EH | $Ap^R$, *tacP* (pSF1010) | Fürste et al. (1986) |
| pEPA53 | *xylE* (2 kb), (pUC19) | Cuskey, S., USEPA, Gulf Breeze |
| pWWO | $Tol^+$, $Xyl^+$ | William and Worsey (1974) |
| NAH7 | $Nah^+$, $Sal^+$ | Grund and Gunsalus (1983) |
| pDC15 | $3Cba^+$ (pAC27) | Ghosal et al. (1985a) |
| pUS1 | $Tc^R$, comPT88$^+$, 25 kb (pCP13) | This study |
| pUS101 | $Tc^R$, comPT88$^-$, 14 kb (pVDZ'2) | This study |
| pUS102 | $Tc^R$, comPT88$^-$, 4.9 kb (pVDZ'2) | This study |
| pUS105 | $Tc^R$, comPT88$^+$, 8 kb (pCP13) | This study |
| pUS1010 | $Tc^R$, comPT88$^+$, 6 kb (pCP13) | This study |
| pUS1014 | $Tc^R$, comPT88$^-$, 6.5 kb (pVDZ'2) | This study |
| pUS1015 | $Tc^R$, comPT88$^-$, 4.5 kb (pVDZ'2) | This study |
| pUS1016 | $Tc^R$, comPT88$^+$, 5.6 kb (pCP13) | This study |
| pUS1028 | $Tc^R$, comPT88$^+$, 6.7 kb (pCP13) | This study |
| pUS1029 | $Tc^R$, comPT88$^+$, 4.3 kb (pCP13) | This study |
| pUS1031 | $Ap^R$, comPT88$^-$, 4.9 kb (pMMB6EH) | This study |
| pUS1032 | $Ap^R$, comPT88$^+$, 4.9 kb (pMMB6EH) | This study |

[a] ComPT88$^+$, complementation of PT88; plasmids in parentheses represent the vectors or replicons used in construction.

pellet of cells (0.5 g) grown in BSM with 2,4,5-T was suspended in 5 ml of 10 mM Tris · HCl pH 8.0 and rapidly mixed with 100 ml of freshly prepared lysis buffer (1% SDS, 1 mM EDTA, 10 mM Tris · HCl pH 12.45), stirred for 5 min at room temperature and incubated for 10 min at 37°C. 2 M Tris · HCl pH 7.0 (20 ml), was added to the lysate at room temperature with stirring for 5 min. Solid NaCl was added to 3% final concentration and the mixture was kept on ice for 1 h. The mixture was extracted with 100 ml ET-saturated phenol followed by 100 ml chloroform–isoamyl alcohol (24 : 1) by stirring for 15 min in each case. Two volumes of cold 95% ethanol were added to the aqueous phase and stored at −20°C overnight. The DNA was pelleted at 5°C, suspended in ET buffer and plasmid DNA purified by EtdBr-CsCl gradient ultracentrifugation.

### (e) Restriction and ligation

DNA fragments were generated by digesting 0.5 to 2.0 μg of DNA in a 20-μl digestion mixture using buffers as described (Davis et al., 1980) and as specified by the manufacturer. Reactions were stopped by

heating at 65°C for 10 min. Ligations were carried out by using T4 DNA ligase (New England BioLabs, Beverly, MA). For preparative digestions 20 μg of plasmid DNA was cut with appropriate amount of restriction enzyme and subjected to electrophoresis on a low melt agarose gel (FMC, Rockland, ME). The desired fragment was placed under minimum UV (long wave) illumination, cut out and purified to remove the residual agarose.

**(f) Agarose and polyacrylamide gel electrophoresis**

Electrophoresis of restriction endonuclease digests was performed in horizontal 0.7 to 1% agarose gels or vertical 6% polyacrylamide gels in TBE buffer (Maniatis et al., 1982). *Hin*dIII fragments of phage λ DNA and 1-kb ladder (BRL, Rockville, MD) were used as $M_r$ standards for gels.

**(g) Transformation**

*E. coli* AC80 strains were rendered competent using a $CaCl_2$ treatment and a cryogenic preservation procedure (Morrison, 1977). Transformations of *E. coli* were carried out as described by Maniatis et al. (1982).

**(h) Construction of clone bank**

*P. cepacia* AC1100 genomic DNA (250 μg) was partially digested with *Bam*HI, under carefully controlled conditions so as to yield fragments of 15 to 30 kb in size (Maniatis et al., 1982). The DNA from such digestion was extracted with phenol and ethanol-precipitated before use. Cosmid vector pCP13 (Table I) was isolated and digested with *Bam*HI to remove the $Km^R$ gene. Linearized cosmid DNA was then dephosphorylated with calf intestinal alkaline phosphatase (Boehringer-Mannheim Biochemicals, Indianapolis, IN). The *Bam*HI fragments of AC1100 DNA (10 μg) were mixed with *Bam*HI digest of pCP13 (2 μg) and were ligated in a total volume of 25 μl with T4 DNA ligase. Ligated DNA was packaged into phage λ particles in vitro using λ packaging kits (Amersham Corp., Arlington Heights, IL) containing extracts from λ lysogenic *E. coli* strains BHB2688 and BHB2690 (Hohn, 1979). The packaging mixture of λ particles (10–50 μl) was added to 200 μl of AC80 cells and incubated for 15 min at room temperature to allow infection and transferred to 3 ml of L broth for 45 min at 37°C, before plating on L agar with 10 μg Tc/ml. All the $Tc^R$ clones, carrying recombinant DNA, were scraped, suspended in Hogness freezing medium (LB supplemented with 8.8% glycerol, 3 mM trisodium acetate, 55 mM $K_2HPO_4$, 26 mM $KH_2PO_4$, 1 mM $MgSO_4$, 15 mM $(NH_4)_2SO_4$; Franklin, 1985) and stored at −70°C.

**(i) Complementation analysis**

To mobilize pCP13-recombinant DNA molecules to Tn*5* mutants of *P. cepacia* triparental matings were performed using the conjugative properties of pRK2013 (Figurski and Helinski, 1979). Hybrid cosmid DNA molecules from the $Tc^R$ and 2,4,5-$T^+$ partial diploids were isolated after growing on 2,4,5-T media for five to six days. Purified cosmid DNA (1 μg) was used to transform competent cells of *E. coli* AC80 to Tc resistance. For routine complementation analysis of different fragments of cloned DNA in *E. coli* AC80, matings with Tn*5* mutants of AC1100 were plated on 2,4,5-T medium and incubated as above.

**(j) Southern-blot hybridization**

Digests of 3 μg of genomic DNA were separated by electrophoresis in 0.8% agarose gels and transferred to nylon membranes (NA45; Schleicher & Schuell, Inc.) by electroblotting in a Bio-Rad transblot cell (Bio-Rad Laboratories, Richmond, CA) at 80 V for 4 h or 400 mA for 2 h. Hybridization was carried out with radiolabeled DNA made by using a nick-translation kit with [α-$^{32}$P]dCTP (Amersham).

## RESULTS AND DISCUSSION

**(a) Tn*5*-mutants**

In previous studies ColE1::Tn*5* was used as transposon delivery system to obtain Tn*5*-insertion mutants which were blocked in the 2,4,5-T-degradative pathway (Tomasek et al., 1986). To identify and localize genes associated with 2,4,5-T degradation we used 50 such insertion mutants which were not

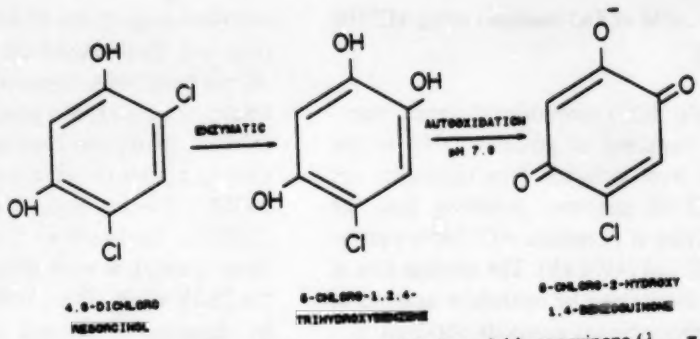


Fig. 1. Mechanism of conversion of 4,6-dichlororesorcinol to 5-chloro, 2-hydroxy-1,4-benzoquinone ($\lambda_{max}$ = 493 nm) by PT88 cells.

readily revertible to the parental 2,4,5-T$^+$ phenotype under selective pressure. The ability of most of these mutants to accumulate various colored intermediates from 2,4,5-T was gradually lost on continued passage of the cultures on media without 2,4,5-T. Mutant PT88, however, was very stable in accumulating a colored intermediate from 2,4,5-T and was therefore used in the present studies.

**(b) Development of an assay system for intermediate accumulation with mutant PT88**

When grown on glucose in presence of 2,4,5-T, PT88 accumulates a bright red compound in the medium as a result of a block in the dissimilatory pathway of 2,4,5-T. This compound has a $\lambda_{max}$ at 493 nm at neutral pH, and the spectral analysis suggested that it is a chlorinated hydroxy-benzoquinone, an autooxidation product of the corresponding chlorohydroxy-hydroquinone (Chapman et al., 1987). Accumulation of a mono-chlorinated trihydric phenol from 2,4,5-trichlorophenol, a previously established 2,4,5-T metabolite (Karns et al., 1983), required displacement of two chlorine atoms by hydroxyl groups. A simple test for the presence in mutant PT88 of enzymes catalyzing chlorine displacement was developed. Washed cells of PT88 grown with glucose and 2,4,5-T act on 1 mM 4,6-dichlororesorcinol to produce the same red colored reaction product shown to be identical with the autooxidation product of CHQ or 5-chloro-1,2,4-trihydroxybenzene (P.J. C., U.M.X. S. and A.M. C., manuscript in preparation) (Fig. 1). Uninduced cells of PT88 and wt AC1100 fail to produce this color and induced cells of AC1100 produce only transient and weak color changes (Fig. 2), showing that mutant PT88 is unable to metabolize 5-chloro-1,2,4-trihydroxybenzene and its accumulation is signalled by the color of its autooxidation product. The rate of accumulation of colored product from 4,6-dichlororesorcinol by induced cells of PT88, as measured by absorption at 493 nm was essentially linear with time. The rate of accumulation was expressed as an increase in absorbance at 493 nm/h/mg dry weight of cells in 5 ml of incubation mixture. An apparent $K_m$ value of 0.33 mM 4,6-dichlororesorcinol was calculated from a Lineweaver-Burke plot of rate measurements vs. substrate concentrations. Thus PT88 is believed to harbor a mutation in a gene whose product is required for further metabolism of 5-chloro-1,2,4-trihydroxybenzene derived from 2,4,5-T.

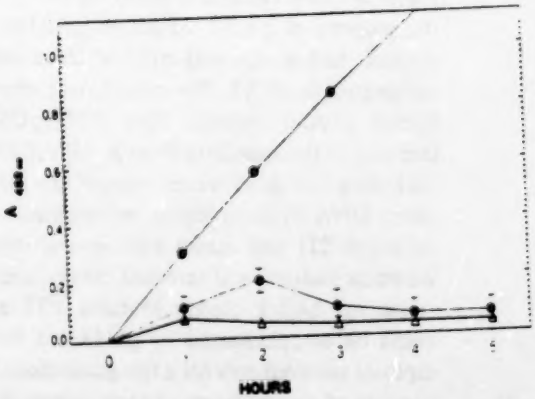


Fig. 2. Complementation assay. Cultures were grown on BSM with glucose and 2,4,5-T. The washed cell suspension was adjusted to $A_{540}$ = 2 with 0.05 M phosphate buffer in 5 ml of incubation mixture containing 1.0 mM 4,6-dichlororesorcinol. Supernatants of aliquots taken during the incubation were analyzed at 493 nm. ●, PT88; △, wt AC1100; ■, PT88 carrying pUS1, pUS105 or pUS1029.

**(c) Complementation of Tn*5*-mutants using AC1100 genomic library**

Approximately 10000 recombinant clones, carrying 20–30-kb fragments of genomic DNA of the strain AC1100 were isolated. This represents ten times the AC1100 genome, assuming that the genomic DNA size of *P. cepacia* AC1100 is equivalent to that of *E. coli* (4100 kb). The average size of the insert was determined by restriction analysis of representative recombinant plasmids obtained.

The pCP13-AC1100-clone bank from *E. coli* AC80 was mobilized into several Tn*5* mutants by triparental mating. The $Tc^R$ transconjugants of each mutant were screened for ability to grow on 2,4,5-T. Only mutant PT88 could be complemented to give stable growth in 2,4,5-T media after six to eight days. Mutants PT4, PT8, PT9, PT14 and PT15 were also complemented to a certain degree, however, they were rather unstable and could not be propagated on 2,4,5-T plates through successive cultures. Twenty 2,4,5-$T^+$ exconjugants of PT88 were grown individually on 500 ml BSM with 2,4,5-T for six to eight days and the hybrid cosmid DNA from each was isolated along with the indigenous plasmids of PT88. After transferring the hybrid cosmids into competent cells of *E. coli* AC80 the $Tc^R$ transformants of each hybrid cosmid were triparentally mated into fresh PT88 cells and spread on 2,4,5-T plates. All such cosmids retained the ability to confer the property of 2,4,5-T utilization on PT88. All the cosmids had an identical insert of 25-kb which was designated as pUS1. The restriction pattern of the hybrid cosmid isolated from PT88[pUS1] was identical to those isolated from *E. coli* AC80[pUS1] indicating that there was no loss of any segment of insert DNA by homologous recombination. *E. coli* AC80[pUS1] was mated with several other Tn*5*-insertion mutants and screened for the complementation on 2,4,5-T plates. Mutants PT8 and PT9 could be complemented by pUS1 but the partial diploids survived only for a few generations possibly because of accumulation of toxic intermediates.

**(d) Subcloning and deletion mapping of the DNA insert**

The cosmid pUS1 which complemented the PT88 mutant was chosen for further characterization. A complete map of the 25-kb insert was constructed (Fig. 3A). To determine the location of 2,4,5-T genes on this long DNA fragment, we constructed a mini library of the smaller segments obtained by *Bam*HI, *Hin*dIII, *Eco*RI and *Xba*I digestion of pUS1 by subcloning into the broad-host-range expression vectors pVDZ'2 (Deretic et al., 1987), pRK290 and pCP13 (Table I). To determine the precise location on the intact gene(s), in vitro deletions were introduced in the 25-kb insert. Thus, various fragments generated by digestion of plasmid pUS1 were recloned in *E. coli*
and transferred to PT88 for complementation (Fig. 3, A and B). Plasmid pUS105 is a result of a deletion of 17-kb region (Fig. 3A). The remaining 8-kb insert complements PT88. Plasmid pUS1010, which also complements PT88, contains a 6-kb insert derived from pUS1 by deletion of *Xba*I fragments (Fig. 3B). By contrast pUS101 in which a 4.9-kb *Bam*HI fragment corresponding to the left-hand portion of the pUS105 insert has been deleted (Fig. 3A) fails to complement PT88. Comparison of the results of complementation by pUS105, pUS101 and pUS102 (which contains only the 4.9-kb *Bam*HI fragment) shows that the genes complementing PT88 are defined by both, the 4.9-kb and the 14-kb *Bam*HI fragments of pUS1. Subcloning these fragments separately destroys their complementing ability. Attempts were made to reduce the size of the insert in pUS1010 by partial digestion with *Eco*RI and recloning in *E. coli*. Only one smaller insert, a 5.6-kb fragment in pUS1016, retained the ability to complement PT88 (Fig. 3B) Plasmids pUS1014 and pUS1015 derived from pUS105 and pUS1010, respectively, by deletion of a 1.5-kb *Bam*HI-*Bgl*II fragment failed to complement, showing that sequences essential for complementation are located at the left end of pUS1016. Both pUS1028 and pUS1029, constructed by deleting the 1.3-kb *Pst*I region from pUS105 and pUS1016, respectively, complemented PT88 (Fig. 3B). However, cloning of the 4.9-kb *Bam*HI fragment in either orientation, failed to complement PT88, indicating that sequences to the right of the *Bam*HI fragment (between *Bam*HI and *Eco*RI sites of pUS1016) are necessary for complementation. The minimum region harboring genes complementing PT88 therefore is spread over a 4-kb *Bam*HI-*Pst*I fragment and a 290-bp segment separated by a 1.3-kb *Pst*I fragment. Since

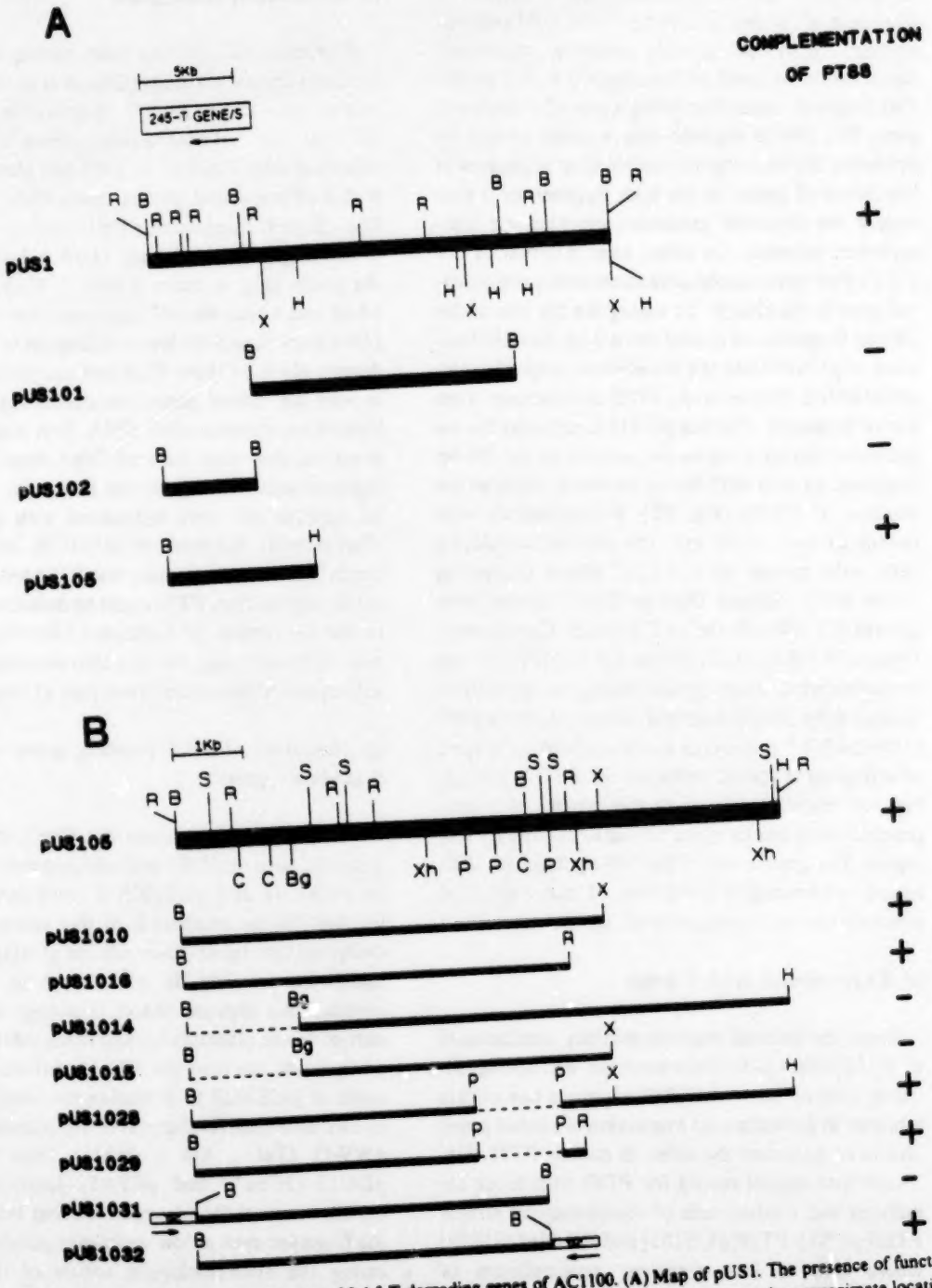


Fig. 3. Restriction map and localization of 2,4,5-T degrading genes of AC1100. (A) Map of pUS1. The presence of functional gene in pUS1 and three sub-fragments was determined by complementation ( + ) of PT88. The box shows the approximate location of 2,4,5-T genes. (B) Extended map of pUS105 and its deletion derivatives. The restriction sites are abbreviated as: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; S, *Sal*I; X, *Xba*I; Xh, *Xho*I. Arrows show direction of transcription originating at $p_{tac}$ promoter (TAC). Dashed lines, deletions.

in general, bacterial structural genes require a minimum of 500 bp, the 290-bp *Pst*I-*Eco*RI segment appears likely to provide essential regulatory functions to the genes on the adjacent 4-kb *Bam*HI-*Pst*I fragment rather than being a part of a structural gene. The 290-bp segment may exercise control by providing the transcription termination sequences to the cluster of genes on the 4-kb fragment or it may supply the promoter sequences essential for transcription initiation. In either case removal of the 1.3-kb *Pst*I region could have eliminated a nonessential gene in the cluster. To determine the role of the 290-bp fragment we cloned the 4.9-kb *Bam*HI fragment of pUS102 into the broad-host-range plasmid, pMMB6HE (Furste et al., 1986) downstream from the *tac* promoter. Plasmid pUS1031 contains the *tac* promoter cloned distal to the position of the 290-bp fragment; in pUS1032 the *tac* promoter replaces the position of 290 bp (Fig. 3B). Both plasmids were mobilized into PT88 and the plasmid-containing cells were spread onto 2,4,5-T plates containing 5 mM IPTG (Sigma). Only pUS1032 allowed slow growth of PT88 cells on 2,4,5-T plates. Complementation of PT88 by pUS1032 but not by pUS1031 can be attributed to transcription having occurred from the end of the 290-bp fragment. However, restoration of the 2,4,5-T$^+$ phenotype at a low efficiency in spite of activation of the *tac* promoter by IPTG is intriguing and might be related to the toxicity of hyperproduction of one or more proteins encoded by this region. The precise role of the 290-bp fragment with regard to its function in the start of transcription is presently under investigation in our laboratory.

**(e) Expression of 2,4,5-T genes**

Since the colored reaction product accumulated by PT88 from 4,6-dichlororesorcinol was not significantly formed by wt AC1100 we made use of this reaction to determine the expression of cloned genes able to complement the defect in mutant PT88. Fig. 2 contrasts typical results for PT88 with those for induced and washed cells of complemented strains PT88[pUS1], PT88[pUS105] and PT88[pUS1029] where only a low, transient accumulation of metabolite is observed similar to that with wt AC1100.

**(f) Localization of the genes**

*P. cepacia* AC1100 has been shown to carry at least two cryptic plasmids (Ghosal et al., 1985b). To locate the cloned 2,4,5-T degradative genes in AC1100, we hybridized electroblots of *Bam*HI digests of total DNA of AC1100 and plasmid DNA with nick-translated probes made from the 4.8-kb *Xba*I-*Bam*HI fragment of pUS1028 (Fig. 3B). None of the fragments of plasmid DNA hybridized with the probe (Fig. 4, lanes 2 and 3). However, both 14-kb and 4.9-kb *Bam*HI fragments of total AC1100 DNA (lane 1) and the identical fragments of *Bam*HI digests of pUS1 (lane 4) lighted up, indicating that at least the cloned genes complementing PT88 are located on chromosomal DNA. It is interesting to point out that when the total DNA from PT88 was digested with *Bam*HI and the fragments, separated on agarose gel, were hybridized with the 4.8-kb *Xba*I-*Bam*HI fragment of pUS1028, none of the bands lighted up, indicating that PT88 has a deletion in this region. Thus PT88 might be defective, not only in the conversion of 5-chloro-1,2,4-trihydroxybenzene to the next step, but may also be defective in the subsequent steps in the lower part of the pathway.

**(g) Homology of 2,4,5-T-coding genes with other degradative genes**

Since PT88 accumulates the CHQ, the 2,4,5-T gene(s) cloned on pUS1 and subsequently subcloned on pUS1028 and pUS1029 is (are) unequivocally involved in the catabolism of this compound. We designate the segment carrying the gene(s) for CHQ metabolism as *chq*. In an attempt to determine whether this segment shares homology with genes carried by the plasmids for degrading other aromatic compounds, we used the 4.8-kb *Xba*I-*Bam*HI fragment of pUS1028 to hybridize the electroblots of *Eco*RI and *Bam*HI digests of the plasmid DNAs, pWWO (Tol$^+$, Xyl$^+$), NAH7 (Nah$^+$, Sal$^+$), pDC15 (3Cba$^+$) and pEPA53 (carrying cloned XylE). None of the plasmids showed homology to the fragment even at low stringency conditions indicating the nonhomologous nature of the cloned 2,4,5-T-coding genes.

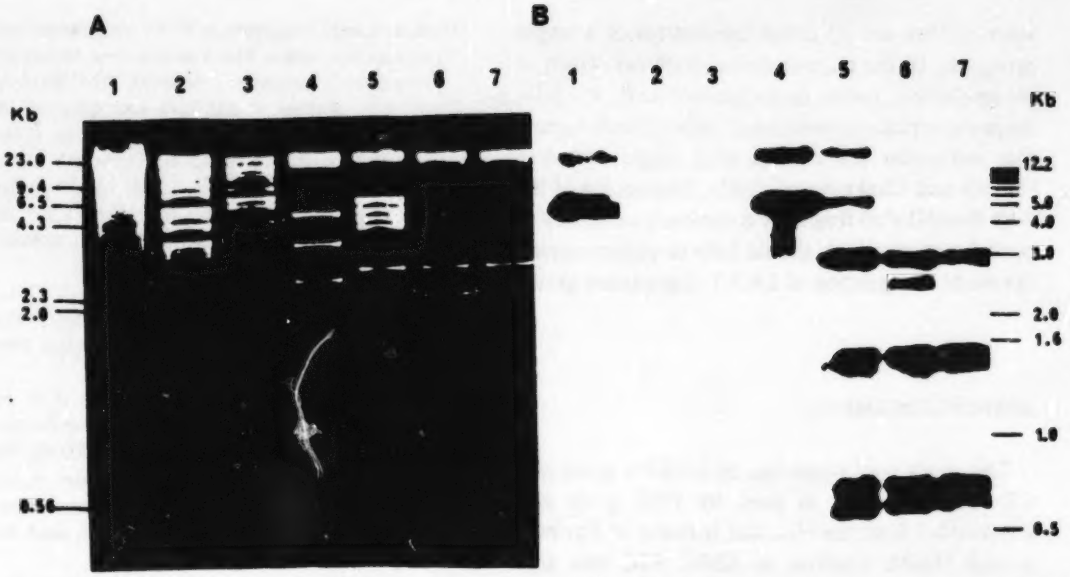


Fig. 4. Localization of 2,4,5-T gene(s) by Southern-blot analysis. Scales on the margin show the positions of size markers. (Panel A) Lanes: 1, total DNA of *P. cepacia* AC1100 digested with *Bam*HI; 2 and 3, plasmid DNA from AC1100 digested with *Bam*HI and *Eco*RI, respectively, 4 and 5, pUS1 digested with *Bam*HI and *Eco*RI, respectively; 6, pUS105 digested with *Eco*RI; 7, pUS1016 digested with *Eco*RI. (Panel B) Autoradiograph of gel in panel A hybridized with $^{32}$P-labeled 4.8-kb *Bam*HI-*Xba*I fragment of pUS1028 (see Fig. 3B).

### (h) Conclusions

There are significant conclusions in this paper that specify the importance of the 2,4,5-T pathway and the genes involved. From soil biodegradation studies using microbial communities, Rosenberg and Alexander (1980) suggested that 2,4,5-trichlorophenol, a product of 2,4,5-T metabolism, is converted to 3,5-dichlorocatechol which is metabolized by the modified *ortho* pathway for chlorocatechols (Frantz and Chakrabarty, 1987). Indeed, we have reported the presence of a variety of chlorocatechols in the growth medium of AC1100 utilizing 2,4,5-T as a sole source of carbon (Ghosal et al., 1985b). The fact that a chlorinated hydroxy-hydroquinone, rather than a chlorinated catechol, is the intermediate of 2,4,5-trichlorophenol degradation, points to the importance of mutational studies in the determination of the pathway of utilization of a compound rather than the accumulation of some compounds in the growth medium, which might be by-products of metabolism. More importantly, recent evidence suggests that diverse microorganisms such as pentachlorophenol-utilizing strains of *Flavobacterium* (Steiert and Crawford, 1986) or the pro-actinomycete *Rhodococcus chlorophenolicus* (Apajalahti and Salkinoja-Salonen, 1987) utilize chlorophenols through chloroquinone and chlorohydroquinone intermediates. Since the 2,4,5-T-degrading *P. cepacia* AC1100 was obtained under strong selective pressure in a chemostat in the presence of 2,4,5-T as its major carbon source, it will be interesting to find out if the chlorohydroxy-hydroquinone degradative genes were recruited from such microorganisms. Since the chlorohydroxy-hydroquinone metabolizing genes are now available as a probe (4.8-kb *Xba*I-*Bam*HI fragment), it would be possible to look for the presence of similar homologous genes in these bacteria.

An interesting facet of the 2,4,5-T degradative gene that encodes further breakdown of the 5-chloro-1,2,4-trihydroxybenzene is the fact that it is chromosomal. Since the 4-kb *Bam*HI-*Pst*I fragment of pUS1029 is absolutely necessary for complementation of PT88 (Fig. 3B), and the flanking 290-bp can be substituted by the *tac* promoter, it is likely that a promoter is present on this fragment and the direction of transcription is from right to left. It is not clear at this stage how many 2,4,5-T degradative genes are present on the *Bam*HI-*Pst*I fragment of pUS1029 or

whether they are all under the control of a single promoter. In the chlorocatechol pathway which is plasmid-borne rather than chromosomal, a 4.3-kb fragment contains three critical chlorocatechol genes that are under the control of a single promoter (Frantz and Chakrabarty, 1987). Sequencing of the 4-kb *Bam*HI-*Pst*I fragment is currently underway in our laboratory which should help in understanding the mode of regulation of 2,4,5-T-degradative genes.

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